Human CAP1 is a key factor in the recycling of cofilin and actin for rapid actin turnover

Kenji Moriyama* and Ichiro Yahara‡

Department of Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan.
*Author for correspondence (e-mail: moriyama@rinshoken.or.jp)
‡Present address: Medical & Biological Laboratories Co., Ltd., 1063-103, Ohara, Terasawaoka, Ina-city, 396-0002, Japan

Accepted 19 January 2002
Journal of Cell Science 115, 1591-1601 (2002) © The Company of Biologists Ltd

Summary

Cofilin-ADF (actin-depolymerizing factor) is an essential driver of actin-based motility. We discovered two proteins, p65 and p55, that are components of the actin-cofilin complex in a human HEK293 cell extract and identified p55 as CAP1/ASP56, a human homologue of yeast CAP/SRV2 (cyclase-associated protein). CAP is a bifunctional protein with an N-terminal domain that binds to Ras-responsive adenylyl cyclase and a C-terminal domain that inhibits actin polymerization. Surprisingly, we found that the N-terminal domain of CAP1, but not the C-terminal domain, is responsible for the interaction with the actin-cofilin complex. The N-terminal domain of CAP1 was also found to accelerate the depolymerization of F-actin at the pointed end, which was further enhanced in the presence of cofilin and/or the C-terminal domain of CAP1. Moreover, CAP1 and its C-terminal domain were observed to facilitate filament elongation at the barbed end and to stimulate ADP-ATP exchange on G-actin, a process that regenerates easily polymerizable G-actin. Although cofilin inhibited the nucleotide exchange on G-actin even in the presence of the C-terminal domain of CAP1, its N-terminal domain relieved this inhibition. Thus, CAP1 plays a key role in speeding up the turnover of actin filaments by effectively recycling cofilin and actin and through its effect on both ends of actin filament.

Key words: Actin, Adenylyl cyclase-associated protein, Cofilin/ADF, Cell motility, Actin-interacting protein 1

Introduction

Actin-based motility, such as lamellipodial protrusion, is driven by the directional elongation of actin filaments (reviewed in Mitchison and Cramer, 1996). In addition to elongation at the barbed end (fast-growing end) of the filament, continuous movement requires efficient recycling of used actin subunits, which is mediated by the release of actin monomers from the pointed end (slow-growing end) of the filament. Although their release is very slow when actin is isolated, cofilin/ADF dramatically accelerates this process at the pointed end and, as a result, enhances the turnover (or treadmilling) of actin filaments (Carlier et al., 1997; Rosenblatt et al., 1997). Thus, cofilin has an essential role in the recycling of actin subunits required for actin-based motility. Cofilin, however, strongly inhibits nucleotide exchange on G-actin (Nishida, 1985; Blanchoin and Pollard, 1998), a process that regenerates readily polymerizable ATP-actin monomers. This property of cofilin presents a serious obstacle to the rapid turnover of actin filaments. It is thought that this obstacle might be avoided by regulation of cofilin-phosphorylation/dephosphorylation in vivo (Rosenblatt and Mitchison, 1998; Chen et al., 2000), because higher eukaryotes possess cofilin-kinases (including LIM kinases) that inhibit cofilin through phosphorylation (Arber et al., 1998; Yang et al., 1998). It is thought that phosphorylation of cofilin causes the release of bound ADP-actin, which is, in turn, converted to free ATP-actin and used in the next round of polymerization.

CAP/Srv2 was first identified in *S. cerevisiae* as a binding partner of adenylyl cyclase, Cyr1, which is activated by a small G-protein, Ras (Field et al., 1990; Fedor-Chaiken et al., 1990). CAP has three domains. The N-terminal domain binds to Cyr1 and is involved in its Ras-responsiveness (Gerst et al., 1991; Mintzer and Field, 1994; Nishida et al., 1998). The C-terminal domain binds to G-actin and strongly inhibits actin polymerization (Freeman et al., 1995). The precise function of the internal, proline-rich domain of CAP is still unclear. Mammals have at least two CAP homologues (CAP1 and CAP2). Both CAP1 and CAP2, like CAP/Srv2, have been reported to bind to G-actin at their C-terminal domains and not at their N-terminal domains (Hubberstey et al., 1996). Recently, a CAP homologue of *Drosophila* has been reported to play a role in developmental morphogenesis, probably through its effect on the actin cytoskeleton (Benlali et al., 2000; Baum et al., 2000).

We found two proteins, p65 and p55, in relative abundance in the actin-cofilin complex of a human cell extract, and we determined p55 to be CAP1. Partial sequencing of the p65 peptides revealed that it is a homologue of actin-interacting protein 1, Aip1 (K.M. and I.Y., unpublished). Several lines of evidence indicate that Aip1 homologues assist cofilin through lateral association with F-actin (Iida and Yahara, 1999; Okada et al., 1999; Aizawa et al., 1999; Rodal et al., 1999). Thus, CAP1 may also support cofilin function. Here, we have characterized human CAP1, and its N- and C-terminal domains, in terms of its ability to regulate actin dynamics and co-operate with cofilin. Our novel findings demonstrate that CAP1 effectively recycles actin and cofilin, thereby allowing a rapid turnover of actin filaments, which is an essential driving force behind cell motility.

Materials and Methods

Plasmid construction

pQE-cof.His or pQEH-cof were used for the bacterial production of porcine cofilin carrying a hexahistidine-tag (His-tag) at the C- or the N-terminus, respectively (Moriyama et al., 1996). Recombinant porcine cofilin without a His-tag was prepared as previously described (Moriyama et al., 1990).

pQE63H was constructed by replacing the *Nco*I/*Bpu*1102 I-portion of pQEH-cof with a polylinker sequence: 5′-CCATGGCTAGC*CATCATCACCACCATCAC*GGCtctagaGTCGACCTGCAGGCATGCAAGCTTCGACCTCGAGGGGGGGCCCGGTACCCGGGGATCCAGATCAGCTTAATTAGCTGAGC-3′, in which the italicized sequence encodes for a His-tag and the lower case letters represent a *Xba*I site. This operation eliminated any cofilin-encoding sequences. Another vector, pHSE63, was made by replacing the *Nco*I/*Xba*I portion of pQE63H with the S-tag-encoding *Nco*I/*Eco*RI-170 bp of pBAC-2cp (Novagen, Madison, WI), in which GAATtctaga was the junction between the *Eco*RI- and *Xba*I sites.

I.M.A.G.E. Consortium human EST clones zr28g06 (encoding CAP1) and au45b04 (encoding CAP2) were obtained from Genome Systems (St. Louis, MO). The 690 bp cDNA fragment encoding the N-terminal domain of CAP1 (CAP1-NT) was substituted for the *Nhe*I/*Bam*HI portion of pQE-cof.His. This product, pQE-CAP1-N229.His, directed the efficient expression of 229 CAP1 N-terminal residues in *E. coli*. The 670 bp fragment encoding the C-terminal domain of CAP1 (CAP1-CT) was transferred into *Xba*I/*Hin*dIII sites of pHSE63, thereby generating pHSE-CAP1Δ254, which directed moderate expression of 221 CAP1 C-terminal residues in *E. coli*.

pUSR-cof.HA and pSRA7-cof have been used to express HA-tagged cofilin in mammalian cells (Moriyama et al., 1996). In the present study, the 690 bp fragment encoding CAP1-NT was substituted for the cofilin-coding region of pUSR-cof.HA in order to make pUSR-CAP1-N229.HA. pUSR-CAP2-N228.HA was constructed in a similar manner. pSRA-CAP1Δ254 was made by replacing the cofilin-coding region of pSRA7-cof with the *Xba*I/*Bam*HI 670 bp fragment of pHSE-CAP1Δ254. pUSH-CAP1 was generated from pUSH-cof, then used for the production of His-tagged CAP1 in HEK293 cells.

cDNAs for human p57^{WD} (coronin), fascin, EF1α and CAP1 were obtained from human U937 mRNA by RT-PCR. Plasmids for mammalian expression of their HA-tagged forms were created in a similar manner to pSRA-CAP1Δ254 and used for the experiment shown in Fig. 1B.

Preparation of cofilin-associated proteins

HEK293 cells were transfected by lipofectamine (Gibco BRL, Grand Island, NY) using a plasmid that drives the expression of Cof-His$_6$, a porcine cofilin carrying a His-tag at its C-terminus. After 2 days, confluent cells were rinsed with Hepes-buffered saline and lysed in lysis solution (20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin). The crude lysate was clarified by centrifugation, quantified for protein concentration using Bio-Rad protein assay reagent and adjusted to 6 mg/ml by diluting with the lysis solution. Then, 0.5 ml of the lysate was incubated with 30 μl of Ni^{2+}-resin to adsorb Cof-His$_6$ and associated molecules. The resin was washed three times with the lysis solution and bound material was eluted by exposure to increasing concentrations of NaCl.

Protein expression and purification

The N- and C-terminal domains of CAP1 were expressed in *E. coli* BL21(Rep4) carrying pQE CAP1-N229.His and pHSE-CAP1Δ254, respectively. CAP1-CT contained both a His-tag and an S-tag at its N-terminus, whereas CAP1-NT possessed only a C-terminal His-tag. The domains produced were purified using a Ni^{2+} column, as previously described for His-tagged cofilin (Moriyama et al., 1996). Full-length CAP1 was prepared from HEK293 cells with a His-tag at its N-terminus. Cells (50 dishes) were transfected with pUSH-CAP1. After 2 days, lysate was prepared and applied to a Ni^{2+} column. The column was washed thoroughly with a 0-800 mM NaCl gradient in 20 mM Tris-HCl, 0.1 mM ATP, 0.05 mM DTT, 0.5 mM PMSF (pH 7.5). Following this, the protein was eluted using a 10-500 mM imidazole gradient in the same buffer. Peak fractions were dialysed and fractionated with a SP-Sepharose column and a 0-700 mM gradient of NaCl. Peak fractions were concentrated and processed in two different columns. One aliquot was gel-filtrated through a Superdex 200pg column (HR16/60), and the other was reapplied onto a Ni^{2+} column. The latter column was washed with 4 M urea, 20 mM Tris-HCl (pH 8) in order to liberate actin that was tightly bound to His$_6$-CAP1. Then, pure His$_6$-CAP1 was eluted with a 0-500 mM gradient of imidazole. Purified CAP1 and its domains were dialysed in 20 mM Tris-HCl, 50 mM KCl, 0.2 mM DTT (pH 7.5). The protein concentration was determined by the method of Gill and von Hippel (Gill and von Hippel, 1989), because CAP1 domains exhibited only a little difference (within 5.9%) in absorbance at 280 nm between native and denatured states.

Antibodies

HSE-CAP1Δ254 (CAP1-CT) was cleaved with enterokinase (Novagen), leading to the release of an N-terminal extra peptide (containing His- and S-tags), which was removed by adsorption to a Ni^{2+} column. Rat antiserum to CAP1-CT was made by Sawady Technology (Tokyo, Japan) and affinity-purified with a CAP1-CT-conjugated Sepharose column. Alkaline-phosphatase-labeled antibody to rat IgG was purchased from ICN Biomedicals (Aurora, OH). Cy3-conjugated anti-rat IgG was obtained from Jackson (West Grove, PA). Rabbit antibodies to actin, cofilin and a mouse antibody to an HA-tag (12CA5) were previously described (Iida et al., 1992; Moriyama et al., 1996).

Subunit exchange assay

Preparation of *N*-(1-pyrene) iodoacetamide-labeled actin (pyrene-actin) and the method of fluorescence measurement were described previously (Moriyama and Yahara, 1999). 6 μM Mg-actin was polymerized in the presence or absence of 18 nM gelsolin. 40 μl of fully polymerized actin was removed and added to 60 μl of 17 mM Tris-HCl, 10 mM Hepes-KOH, 2 mM $MgCl_2$, 100 mM KCl, 0.5 mM EGTA, 0.1 mM DTT (pH 7.2) containing the CAP1-domains and/or cofilin. Then it was immediately mixed with 20 μl of 1.2 μM pyrene-actin monomer (50%-labeled), which had been converted to its Mg-ATP-bound form just before being added. The incorporation of pyrene-actin into unlabeled F-actin was monitored by a Perkin-Elmer LS-50B Luminescence spectrophotometer.

Dilution-induced depolymerization assay

10 μM Mg-actin (10% pyrene-labeled) was polymerized in the presence or absence of 40 nM gelsolin over the course of 4 hours. Depolymerization was initiated by a 20-fold dilution with 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 100 mM KCl, 0.5 mM EGTA, 0.1 mM DTT containing the CAP1-domains and/or cofilin. The decline in fluorescence was immediately monitored. The dilution solution for the gelsolin-capped filaments was supplemented with 2 nM gelsolin-actin complex to cap the barbed ends.

Observation of actin turnover kinetics

The kinetics of actin filament turnover (treadmill) were observed using 1,N^6-etheno ATP (εATP)-labeled F-actin, which was prepared according to the procedure of Didry et al. (Didry et al., 1998). 6 μM

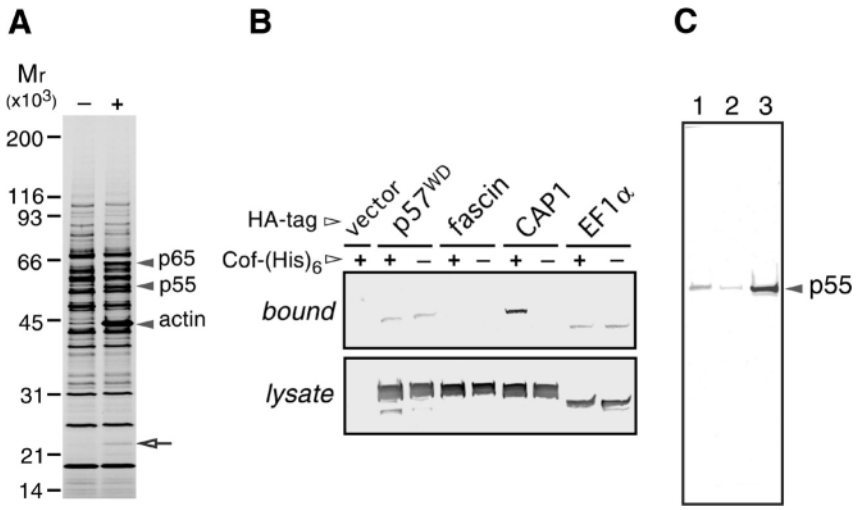

Fig. 1. Identification of human cellular proteins associated with the cofilin-actin complex. (A) HEK293 cells were transfected with either a plasmid that drove the expression of Cof-His_6 (lane +), or a control vector (lane –). Cell lysate (3 mg protein equivalent) was incubated with Ni^{2+}-NTA resin to adsorb Cof-His_6 and associated proteins. Bound material was eluted with step-wise increments in the concentration of NaCl. The eluate was electrophoresed on a 10-20% polyacrylamide gradient gel and silver stained. The lanes of the 300 mM NaCl eluates ares shown. The arrowheads point to bands that were recovered specifically from Cof-His_6-producing cells. A small amount of Cof-His_6 leaked out of the resin as a result of NaCl washing (arrow). (B) HA-tagged actin-binding proteins ($p57^{WD}$, fascin, CAP1 and EF1α) were expressed separately in HEK293 cells with (+) or without (–) co-expression of Cof-His_6. Cof-His_6-associated protein fractions were prepared as above. Crude lysates (lysate) and 500 mM NaCl eluates (bound) were subjected to western blotting with an anti-HA MAb (clone 12CA5). The size of the bands is around 55kDa. (C) Anti-CAP1 blotting of p55. Crude lysate (lane 1) and 300 mM NaCl eluates from Ni^{2+}-resin (lanes 2 and 3) were subjected to western blotting with an affinity-purified rat IgG to CAP1. The NaCl eluate from Cof-His_6-producing cells is clearly positive for the CAP1 signal (lane 3), in contrast to the eluate from non-expressing cells (lane 2).

εATP-actin was converted to its Mg-bound form and was polymerized in the presence or absence of the CAP1 domains and/or cofilin in 10 mM Tris-HCl, 7 mM Hepes-KOH, 2 mM $MgCl_2$, 100 mM KCl, 20 μM εATP, 0.5 mM EGTA, 0.1 mM DTT (pH 7.1). After a steady state (apparent equilibrium) was reached, ATP was added at a final concentration of 0.25 mM, and the fluorescence was recorded by measuring emission at 410 nm and excitation at 360 nm.

Nucleotide exchange reaction on G-actin

15 μM of Ca-actin was incubated in 80 μM $MgCl_2$ with 0.2 mM EGTA in G-buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM DTT, pH 7.8) for 5 minutes and then reacted with 20 units/ml of hexokinase (Sigma, St. Louis, MO) and 0.3 mM D-glucose. After 2 hours at 4°C, free nucleotides were removed with a Dowex1-X8 ion exchange resin. The remaining 12 μM of Mg-ADP-actin was supplemented with 36 μM ADP. 10 μl of Mg-ADP-actin monomer was mixed with an equal volume of 18 μM cofilin or its solvent for 3 minutes. Then, 100 μl of 60 μM εATP solution containing the CAP1 domains was added, and the increase in fluorescence was recorded. The reaction buffer contained 20 mM Hepes-KOH, 8 mM Tris-HCl, 2 mM $MgCl_2$, 80 mM KCl, 0.1 mM EGTA and 0.1 mM DTT (pH 6.9).

Other methods

Co-sedimentation with F-actin, His-tag pull-down assay and gel densitometry were performed as described by Moriyama and Yahara (Moriyama and Yahara, 1999). The methods used for western blotting and immunostaining of fibroblasts were previously described by Moriyama et al. (Moriyama et al., 1996).

Results

Human CAP1 associates with a cofilin-actin complex

In a previous study aimed at identifying the phosphorylation site of porcine cofilin (Moriyama et al., 1996), we recovered three proteins bound to His-tagged cofilin using Ni^{2+}-nitrilotriacetic acid (NTA) resin from an extract of HEK293, a human kidney-derived cell line. An immunoblot with anti-actin antibody indicated that one of the three proteins was actin, as expected (data not shown). We designated the other two proteins as p55 and p65 on the basis of their mobility on the SDS polyacrylamide gel (Fig. 1A). Because mutant

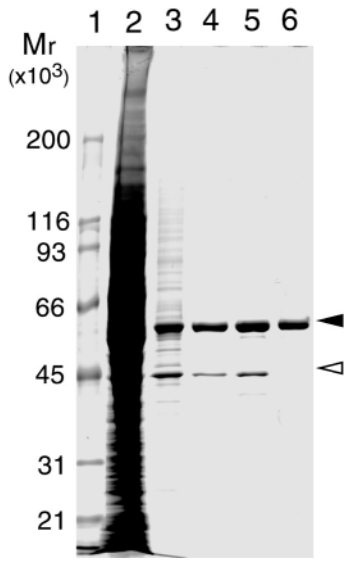

Fig. 2. Purification of His_6-CAP1. HEK293 cells were transfected with pUSH-CAP1. The cell lysate was prepared (lane 2), and His_6-CAP1 was successively enriched upon a Ni^{2+}-NTA- (lane 3) and a SP-Sepharose column (lane 4). Although gel filtration through a Superdex 200 column could not separate bound G-actin from His_6-CAP1(lane 5), bound actin was liberated by treating the Ni^{2+}-column-bound His_6-CAP1-actin complex with urea. Pure His_6-CAP1 was then eluted with imidazole after the urea was removed (lane 6). Molecular size standards were run on lane 1.

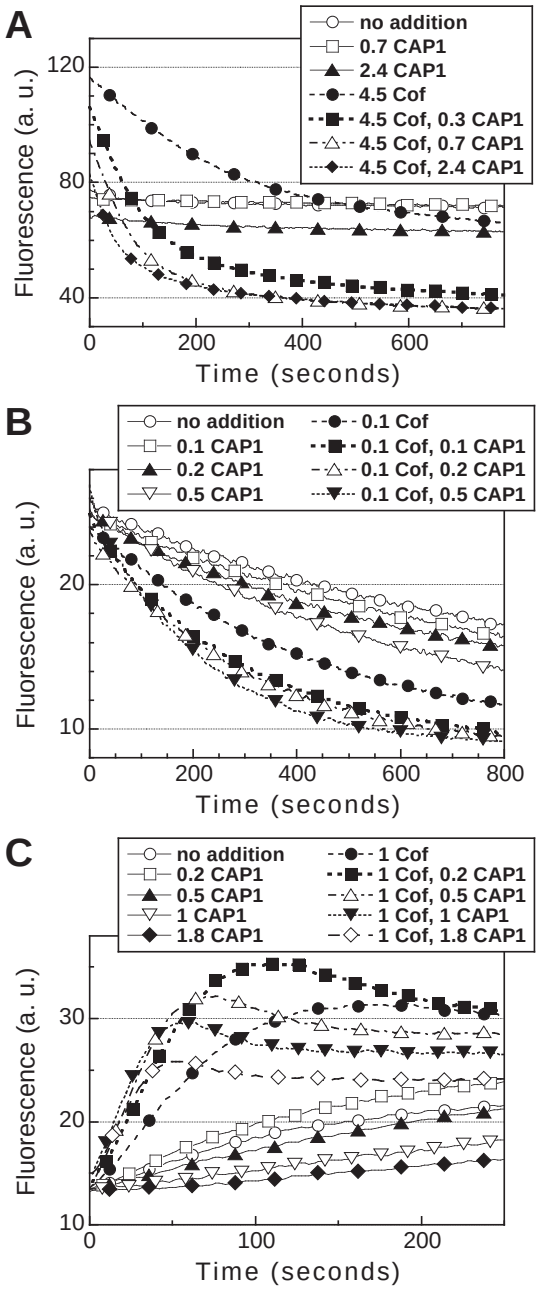

Fig. 3. Effects of human CAP1 on actin dynamics. (A) His_6-CAP1 promotes the turnover of F-actin in the presence of cofilin. 6 μM 1,N^6-etheno ATP (εATP)-actin was polymerized with or without cofilin and/or His_6-CAP1. The intensity of εATP fluorescence is higher when it is bound to actin (Wang and Taylor, 1981). After a steady state was reached, excess unlabeled ATP was added to chase actin-bound εATP, and the decline in fluorescence was recorded. Although binding of cofilin to εATP-F-actin was observed to increase the basal intensity of fluorescence, it was possible to semi-quantitatively evaluate CAP1 activity. The legends of each graph state the protein concentration in μM. 'Cof' designates bacterially produced porcine cofilin without a His-tag. (B) Effect of His_6-CAP1 on the rate of actin depolymerization. Gelsolin-capped actin filaments (10%-pyrene labeled) were diluted 20-fold in the presence or absence of His_6-CAP1 and/or cofilin. The fluorescence intensity was recorded to monitor gradual depolymerization. Pyrene-labeled actin is more fluorescent when it is in F-actin than in its unpolymerized state (Kouyama and Mihashi, 1981). (C) Subunit exchange assay using F-actin seeds with free barbed ends. Unlabeled F-actin was mixed with cofilin and/or His_6-CAP1, then a small amount of pyrene-labeled Mg-G-actin was immediately added. The incorporation of pyrene-actin into unlabeled F-actin was monitored by the change in fluorescence. Final concentrations of pyrene-actin and unlabeled actin were 0.2 μM and 2.0 μM, respectively.

cofilins lacking actin-binding activity did not recover either of the two proteins, they were presumed to interact primarily with actin. To identify p55, we selected four actin-binding proteins with similar molecular sizes as the candidates. The selected proteins were $p57^{WD}$ of the coronin-family (Suzuki et al., 1995), fascin (Duh et al., 1994), CAP1/ASP56 and EF1α (Brands et al., 1986). His-tagged cofilin was co-expressed with each of the four proteins carrying an HA-epitope tag at their N-termini in HEK293 cells. Whole cell lysate and His_6-cofilin-bound fractions were prepared and subjected to western blotting with an anti-HA antibody (Fig. 1B). The results suggested that the band of p55 contained CAP1. Thus, we prepared an antibody to human CAP1 and verified that CAP1 was actually present in the His_6-cofilin-bound fraction (Fig. 1C).

CAP1 dramatically enhances the effects of cofilin on actin dynamics

We expressed His-tagged CAP1 in HEK293 cells and purified it by column chromatography (Fig. 2). Gel filtration through a Superdex 200 column suggested that CAP1 exists in a large complex (possibly an oligomer) because it eluted between the elution positions of ferritin (440 kDa) and thyroglobulin (669 kDa). Some of the CAP1 molecules were tightly associated with actin at this step (Fig. 2, lane 5). At the second round of chromatography on a Ni^{2+} column, the bound actin was eliminated by washing with urea, and pure His_6-CAP1 was obtained after renaturation (Fig. 2, lane 6). When the purified His_6-CAP1 was gel filtered as above, it also eluted between ferritin and thyroglobulin even in the absence of actin, further suggesting that CAP1 is oligomeric (data not shown).

The effect of His_6-CAP1 on cofilin-induced changes in actin dynamics was examined in the following experiments (Fig. 3A-C). One of the unique functions of cofilin is the remarkable acceleration of the turnover (or treadmilling) of actin filaments (Carlier et al., 1997; Rosenblatt, 1997; Lappalainen, 1997). In the presence of cofilin, the steady-state turnover of F-actin was dramatically accelerated by CAP1 (Fig. 3A). The faster turnover of F-actin with cofilin is primarily brought about by its activity to speed up depolymerization at the pointed end of actin filament (Carlier et al., 1997; Moriyama and Yahara, 1999). CAP1 moderately stimulated the depolymerization of gelsolin-capped actin filaments both in the presence and absence of cofilin (Fig. 3B). 0.5 μM of CAP1 increased the apparent rate constant for depolymerization by 1.7-fold in the absence of cofilin and 1.9-fold in its presence (Fig. 3B). In addition to such stimulation of subunit release at the pointed end, we found that CAP1 promoted the cofilin-enhanced turnover of F-actin by facilitating actin assembly at the barbed end of actin filament. CAP1 increased the maximal rate of incorporation of actin monomers into pre-existing actin filaments in the presence of cofilin (Fig. 3C; Fig. 5E). These results clearly contrast with those of a number of studies

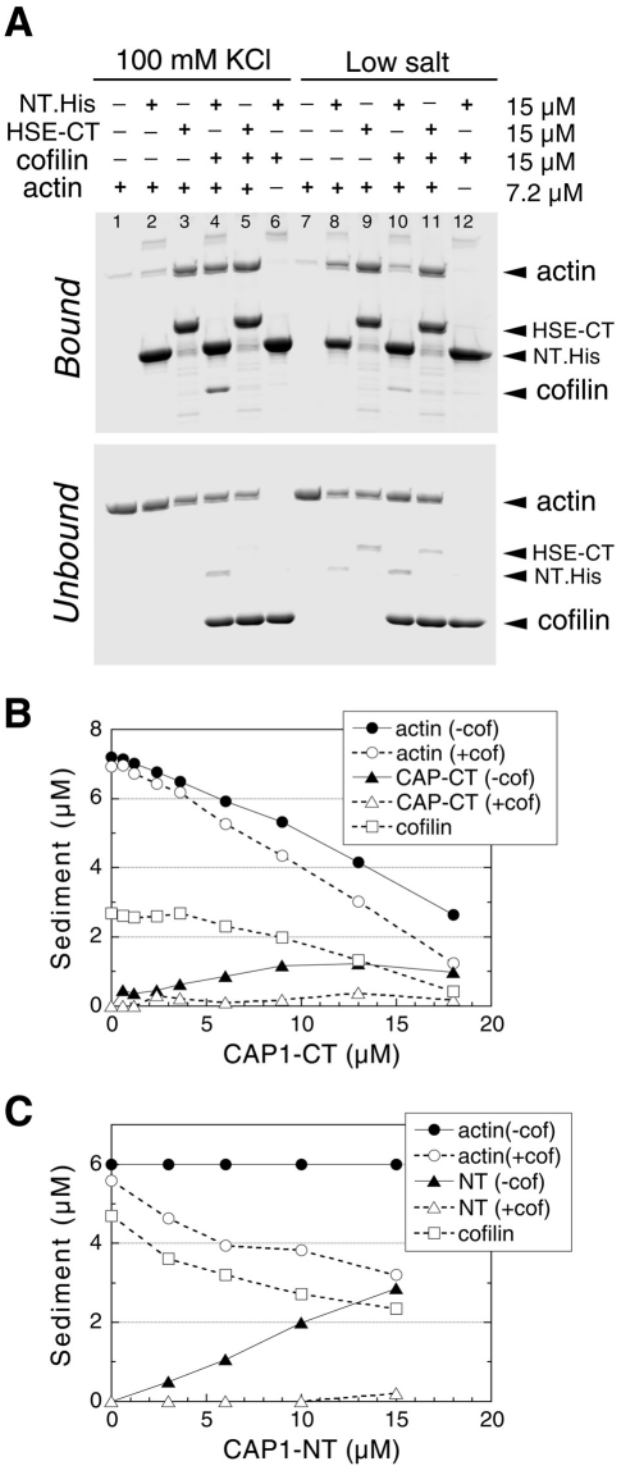

Fig. 4. The N-terminal domain of CAP1 is responsible for its cofilin-dependent interaction with unpolymerized actin. (A) The cofilin-actin complex interacts with CAP1-NT but not with CAP1-CT. G-actin (29 μM) was converted to the Mg-ATP-bound form by a 5 minute incubation in 40 μM $MgCl_2$ plus 0.5 mM EGTA. 15 μM of each His_6-tagged CAP1 domain was mixed with 7.2 μM of G-actin, 15 μM cofilin, or both, in a physiological salt buffer (lanes 1-6) or in a low salt buffer (lanes 7-12) at 2°C. Then, His_6-tagged proteins and associated molecule(s) were adsorbed to a Ni^{2+}-resin. The resin was washed four times with 10 mM Tris-HCl, 1 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.1 mM ATP, 0.1 mM DTT (pH 7.5), then boiled in SDS sample buffer. The bound and unbound fractions were analyzed by SDS-PAGE. NT-His designates the His_6-tagged N-terminal domain (amino acids 1-229) of CAP1, and HSE-CT designates the C-terminal domain (amino acids 255-475) carrying both His_6- and S-tags. (B,C) Co-sedimentation of the CAP1 domains with F-actin. F-actin (7.2 μM for B and 6 μM for C) was reacted with various amounts of CAP1-CT (B) or CAP1-NT (C) in the presence (+ cof) or absence (– cof) of cofilin (4 μM cofilin for B and 6 μM for C). After 6 hours, the samples were centrifuged at 190,000 ***g*** for 20 minutes with a Beckman TLA100 rotor. The supernatant and precipitate were subjected to SDS-PAGE. The amount of sedimented protein was quantified with a densitometer and plotted as a function of the amount of the respective CAP1 domains. Both of the CAP domains did not sediment in the absence of F-actin (data not shown).

concerning CAP1 or CAP homologues (Gieselmann and Mann, 1992; Freeman et al., 1995; Gottwald et al., 1996), because they have reported that CAP homologues are strong inhibitors of actin polymerization. The cause of this discrepancy is implicated, in part, in the results shown in Fig. 3C. We noticed that CAP1 had a biphasic effect on the rate of subunit incorporation into F-actin as a function of concentration in the presence of cofilin, that is, a dose-dependent increase was observed up to 1 μM, but higher concentrations of CAP1 were less effective (Fig. 3C; Fig. 5E). Furthermore, such a biphasic pattern of stimulation was observed even in the absence of cofilin when the amount of CAP1 was decreased. The optimal concentration of CAP1 was lower (~0.2 μM) than that observed in the presence of cofilin (Fig. 3C; Fig. 5E). Thus, high concentrations of CAP1 inhibit subunit incorporation into F-actin. This may account for some of discrepancy between our results and those of previous studies (Gieselmann and Mann, 1992; Freeman et al., 1995). CAP1 did not facilitate subunit incorporation into gelsolin-capped filaments and rather prevented it in the absence of cofilin (data not shown), indicating that CAP1-facilitated assembly of actin occurred solely at the barbed end of actin filament and that CAP1 does not sever actin filament. These properties of CAP1 are qualitatively similar to those of profilin, as discussed later.

The N-terminal domain of CAP1 mediates its cofilin-dependent interaction with actin

According to the available literature, CAP homologues exert their effects on actin exclusively by their C-terminal domains. We expressed both domains of CAP1 in *E. coli*, purified each one and examined their activity. A simple binding assay verified an association between the C-terminal domain of CAP1 (CAP1-CT) and actin (Fig. 4). The complex of actin and CAP1-CT bound tightly to DNase I (data not shown), indicating that the bound actin was G-actin, consistent with previous reports. Neither the cofilin-actin complex nor actin-free cofilin were, however, bound by CAP1-CT, although p55 was originally found within a complex containing both cofilin and actin (Fig. 1). This suggests that another region of CAP1 might mediate binding with the complex. The N-terminal domain (CAP1-NT) did not bind G-actin efficiently in the absence of cofilin, whereas the CAP1-CT did (Fig. 4A).

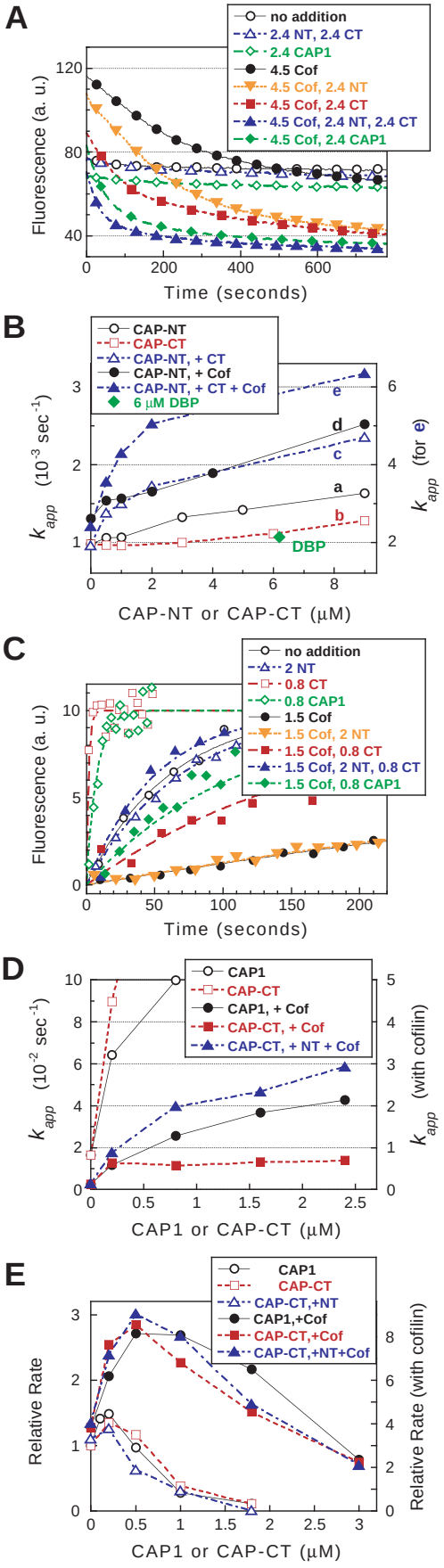

Fig. 5. Effects of the N- and C-terminal domains of human CAP1 on actin dynamics. (A) Effect of the CAP1 domains on the turnover of F-actin. εATP-actin was polymerized as in Fig. 3A. After a steady state was reached, unlabeled ATP was added and the decline in fluorescence was recorded. 2.4 CT and 2.4 NT represent 2.4 μM CAP1-CT and 2.4 μM CAP1-NT, respectively. (B) Effect of the CAP1 domains on the rate of actin depolymerization. Gelsolin-capped actin filaments (10%-pyrene labeled) were diluted in a solution containing the CAP1 domains with or without 0.1 μM of cofilin. When both CAP1 domains were simultaneously used, CAP1-NT was added at a final concentration of 1 μM (triangles, plots c and e). The gradual depolymerization was monitored as in Fig. 3B. The apparent rate constant (k_{app}) of each curve was calculated and plotted as a function of the amount of the respective CAP1-domains. 6 μM vitamin-D-binding protein (DBP) was used as a control actin-sequestering protein, which does not affect the depolymerization rate constant at the pointed end (Weber et al., 1994). (C,D) Effect of CAP1 domains on the rate of nucleotide exchange on G-actin. Mg-ADP-actin was mixed with cofilin or its solvent for 3 minutes. Then, εATP and CAP1 domains were simultaneously added and the exchange of actin-bound ADP to εATP was monitored as fluorescence increased. Final concentrations of actin, cofilin, CAP1-NT and εATP were 1 μM, 1.5 μM, 2 μM and 50 μM, respectively. The exponential curve was fitted for each trace and drawn in the graph C. The apparent rate constants (k_{app}) of exchange reactions were calculated and plotted as a function of the amount of CAP1 or CAP1-CT (D). (E) Subunit exchange assay using F-actin seeds with free barbed ends. Unlabeled F-actin was mixed with cofilin and/or the CAP1 domains, then a small amount of pyrene-labeled Mg-G-actin was immediately added. The incorporation of pyrene-actin into unlabeled F-actin was monitored as in Fig. 3C. The maximal rate (near initial rate) of each fluorescence trace was derived, normalized and plotted as a function of the amount of CAP1 or CAP1-CT. The results with the use of 1 μM cofilin were plotted by filled symbols, and those with the use of 1 μM CAP1-NT were shown by triangles.

Surprisingly, when cofilin was present, CAP1-NT bound both actin and cofilin effectively, whereas CAP1-CT bound only cofilin-free G-actin under physiological ionic condition (Fig. 4A, lanes 1-6). Cofilin did not directly associate with CAP1-NT in the absence of actin, which suggests that cofilin strengthens the otherwise weak association between unpolymerized actin and CAP1-NT.

The association of CAP1 domains with F-actin was assessed by examining their co-sedimentation with polymerized actin (Fig. 4B,C). In the absence of cofilin, both N- and C-terminal domains of CAP1 slightly sedimented with F-actin. However, co-sedimentation of CAP1-NT and CAP1-CT with F-actin was almost non-existent following the inclusion of cofilin (Fig. 4B,C). The possible oligomeric nature of the full-length CAP1 made it difficult to evaluate its lateral association with F-actin (the CAP1-actin complex obtained from the Superdex column step was partially sedimented by analogous centrifugation). CAP1-CT efficiently decreased the amount of sedimentable F-actin both in the presence or absence of cofilin (Fig. 4B). Interestingly, CAP1-NT decreased the amount of sedimentable F-actin in the presence of cofilin but not in its absence (Fig. 4C).

Novel roles for the N- and C-terminal domains of CAP1 in stimulating cofilin-enhanced actin dynamics

The effect of each CAP1 domain on actin dynamics was examined. As well as full-length CAP1, both CAP1-NT and

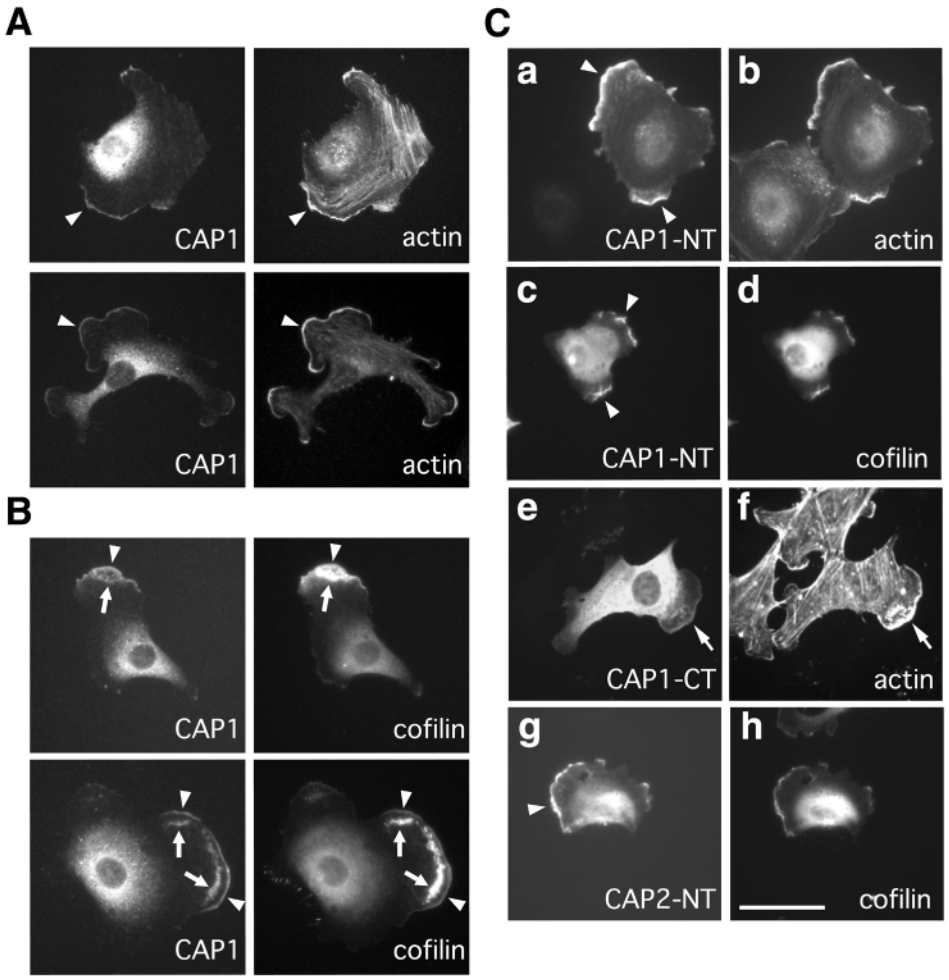

Fig. 6. Colocalization of CAP1 with cofilin, and analysis of factors that determine its localization. (A,B) Spreading mouse C3H-2K fibroblasts were fixed and incubated with a rat anti-CAP1 IgG and a rabbit antibody to actin (A) or cofilin (B), followed by labelling with a Cy3-labeled anti-rat IgG and a fluorescein-labeled anti-rabbit IgG. The lamellipodia are indicated by arrowheads, whereas the ruffling areas are indicated by arrows. (C) HA-tagged CAP1-NT was transiently expressed in C3H-2K cells (panels a-d) by lipofection. HA-tagged CAP1-CT (panels e and f) or HA-tagged CAP2-NT (panels g and h) were expressed as well. Transfected cells were split onto coverslips, and the spreading cells were fixed and reacted with a mouse anti-HA and a rabbit antibody to actin (panels a, b, e and f) or to cofilin (panels c, d, g and h), after which they were stained with a Cy3-labeled anti-mouse IgG and a fluorescein-labeled anti rabbit IgG. The lamellipodia are indicated by arrowheads (for CAP-NTs) or arrows (for CAP1-CT). The white bar represents 10 μm.

CAP1-CT enhanced cofilin-induced acceleration of F-actin turnover, whereas their effect was only marginally visible in the absence of cofilin (Fig. 5A). It was noted that either CAP1-NT or CAP1-CT exhibited weaker enhancement of cofilin activity than full-length CAP1, but the simultaneous addition of both domains produced activity almost equal to that of full-length CAP1 (Fig. 5A). CAP1-NT also increased the depolymerization rate of gelsolin-capped actin filaments, which was faster in the presence of cofilin and/or CAP1-CT (Fig. 5B). This effect was significant even in the absence of cofilin (Fig. 5B), suggesting a direct interaction of CAP1-NT with the pointed end of actin filament.

We found another novel activity of CAP1. It dramatically accelerated nucleotide exchange on G-actin, a process to regenerate readily polymerizable ATP-actin (Fig. 5C,D). The C-terminal domain of CAP1 had a similar effect (Fig. 5C,D). The opposite effect was reported for cofilin/ADF (Nishida, 1985; Blanchoin and Pollard, 1998). In fact, cofilin remarkably delayed the nucleotide exchange (Fig. 5C). This property of cofilin must pose a serious obstacle to the rapid turnover of actin filaments. With regard to cofilin-bound G-actin, CAP1-CT showed weaker nucleotide exchange activity than CAP1 (Fig. 5C,D). CAP1-NT did not affect the rate of nucleotide exchange in the absence of CAP1-CT, even when cofilin was present (Fig. 5C). In addition, it did not show any effect on the acceleration observed with CAP1-CT in the absence of cofilin (data not shown). When G-actin was complexed with cofilin, however, CAP1-NT enhanced the stimulative effect of CAP1-CT and reproduced almost similar effects to those obtained with full-length CAP1 (Fig. 5C,D). Therefore, CAP1 promotes actin dynamics not only by enhancing the stimulatory effects of cofilin with regard to actin turnover but also by reducing its inhibitory effect.

CAP1 facilitated the assembly of actin monomers onto the barbed end of pre-existing actin filaments (Fig. 3C). The same experiment was carried out with the CAP1 domains, and the maximum rates of subunit incorporation were derived (Fig. 5E). 0.2-0.5 μM of CAP1-CT increased the maximal rate of incorporation of actin monomers into pre-existing actin filaments in the absence of cofilin. In the presence of cofilin, a larger increase was observed, up to 1.8 μM of CAP1-CT. The biphasic profile of this effect with CAP1-CT resembled that obtained with full-length CAP1 (Fig. 5E). 1 μM of CAP1-NT did not obviously affect this activity of CAP1-CT (Fig. 5E), but a much larger amount of CAP1-NT inhibited the subunit incorporation into F-actin either in the presence or absence of CAP1-CT and/or cofilin (data not shown). Thus, CAP1-NT cannot enhance cofilin-induced fragmentation of actin filaments nor facilitate elongation of them.

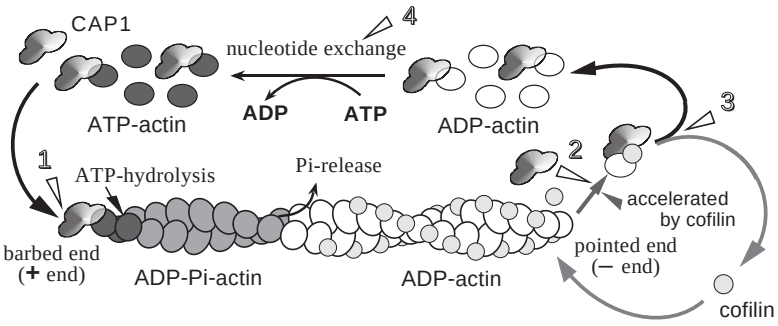

Fig. 7. A schematic model of co-operation between CAP1 and cofilin in promotion of actin dynamics. The working steps of CAP1 are indicated by open arrowheads. (1) CAP1 facilitates the addition of Mg-ATP-actin monomer onto the barbed end of actin filament. Cofilin-induced severing also contributes to this step by increasing the number of barbed ends. (2) CAP1 accelerates subunit release at the pointed end and enhances the more potent, analogous effect of cofilin. (3) CAP1 relieves the inhibitory effect of cofilin on nucleotide exchange of ADP-actin. (4) CAP1 accelerates nucleotide exchange on G-actin.

CAP1 colocalizes with cofilin and actin in dynamic regions of spreading cells

Immunofluorescent staining of spreading fibroblasts showed colocalization of CAP1, cofilin and actin in dynamic regions of the cells (Fig. 6A,B). CAP1 was present with actin in the lamellipodia, but little was observed on the stress fibers (Fig. 6A). It also existed with cofilin in the lamellipodia and in the dorsal ruffles (Fig. 6B). CAP1 was also diffusely present in the inner cytoplasm, as was cofilin.

Next, we expressed CAP1-NT or -CT carrying an HA1-epitope tag in fibroblasts and examined the localization of each domain (Fig. 6Ca-f). CAP1-NT clearly colocalized with actin and cofilin in the lamellipodia (Fig. 6Ca-d). In contrast, CAP1-CT was primarily distributed diffusely throughout the cytoplasm and only weaker staining was visible even in the actin-rich area of lamellipodia (Fig. 6Ce,f; arrow). Thus, the N-terminal domain is primarily responsible for accumulating CAP1 with cofilin and actin in dynamic peripheral regions of spreading cells.

In addition to CAP1, another isoform, CAP2, exists in mammals (Yu et al., 1994, Swiston et al., 1995). We examined the localization of human CAP2-NT and found that it also colocalized with cofilin in the lamellipodia (Fig. 6Cg,h). Our recent biochemical study has also verified that the properties of CAP2-NT are very similar to those of CAP1-NT, with particular reference to the association with the cofilin-actin complex (K.M. and I.Y., unpublished).

Discussion

Novel modes of interaction between human CAP1 and actin

CAP1 and CAP homologues have been characterized as potent inhibitors of actin polymerization (Gieselmann and Mann, 1992; Freeman et al., 1995; Gottwald et al., 1996). Domain-mapping studies have attributed their actin-binding activity to the C-terminal domain (Freeman et al., 1995; Gottwald et al., 1996; Hubberstey et al., 1996; Zelicof et al., 1996). In addition, some reports have demonstrated that the C-terminal domains of several CAP homologues bind to G-actin and thereby inhibit actin polymerization (Freeman et al., 1995; Gottwald et al., 1996). Our novel finding shows that either CAP1 or CAP1-CT can facilitate the addition of G-actin onto the barbed end of actin filament, although inhibition occurs at higher concentrations (Fig. 5E). The optimal amount of CAP1 for the recruitment of G-actin increased remarkably when cofilin was present. At the same time, cofilin broadened the effective concentration range of CAP1. The observed effects of cofilin must be caused by its ability to sever actin filaments, which increases the number of barbed ends where rapid addition of subunits occurs. Previous reports might have overlooked this important consideration when examining CAP1 and CAP homologues because of the narrow effective concentration range in the absence of cofilin. In addition, many studies have used Ca-ATP-actin instead of Mg-ATP-actin, which is the predominant form of actin in living cells.

This is the first report to reveal the association between actin and the N-terminal domain of CAP. Interestingly, this interaction is dramatically enhanced by cofilin and is responsible for CAP1 binding to the cofilin-actin complex. Previous studies have failed to observe the association between actin and the N-terminal domain of CAP1 or CAP homologues because of the cofilin-dependent nature of the interaction. Cofilin prevents several actin-binding molecules, such as tropomyosin and phalloidin, from associating with F-actin by altering the twist of the actin filaments (McGough et al., 1997). In contrast, Aip1 homologues prefer cofilin-bound F-actin as its target (Iida and Yahara, 1999; Okada et al., 1999; Aizawa et al., 1999; Rodal et al., 1999). The actin-binding property of CAP1 is different from other known actin-binding molecules. Cofilin strengthens the association between the CAP1-NT and unpolymerized actin, while reducing the basal weak association of CAP1 domains with F-actin (Fig. 4). It may also be possible that the observed co-sedimentation of CAP1 domains with F-actin was due to non-specific trapping by the F-actin pellet. Our results strongly argue that CAP1-NT associates primarily with the binary actin-cofilin complex, but it remains to be determined whether CAP1-NT associates with other forms of the complex containing three or more molecules of actin and/or cofilin.

The intracellular localization of CAP1 was consistent with its biochemical properties. It is present in dynamic regions of the cell periphery, which are also enriched with both actin and cofilin. Interestingly, CAP1-NT is primarily responsible for the colocalization of CAP1 with actin and cofilin. Thus, it is possible that association of the N-terminal domain of CAP1 with the actin-cofilin complex determines CAP1 localization because the cofilin-actin complex was recognized specifically by the CAP1-NT but not by the CAP1-CT (Fig. 4A). Further analysis of CAP2-NT showed that the actin-modulating function of the N-terminal domain is conserved between human CAP1 and CAP2 (K.M. and I.Y., unpublished). Noegel et al. (Noegel et al., 1999) reported that the enriched localization of a *Dictyostelium* CAP homologue at anterior and posterior edges of cells required its N-terminal domain but not

its C-terminal one. Thus, interaction of CAP-NT with actin may occur in this organism, too, and possibly be conserved in different species.

Synergistic effects of CAP1 and cofilin in accelerating actin dynamics

A rapid turnover of actin filaments is essential for actin-based cell motility, and cofilin-ADF accelerates this turnover (Carlier et al., 1997; Rosenblatt et al., 1997; Lappalainen et al., 1997). Our novel findings illustrate several mechanisms behind the rapid turnover observed in the presence of both CAP1 and cofilin. CAP1 greatly enhances the effect cofilin has on the turnover rate of actin filaments (Figs 3, 5). Our detailed dissection of this effect revealed that it is brought about by four distinct activities, as illustrated in Fig. 7. First, CAP1 facilitates the addition of ATP-actin monomers onto the barbed end of actin filament, an effect that is more pronounced in the presence of cofilin. This effect is biphasic and is a function of the concentration of CAP1 and mediated by its C-terminal domain (Fig. 5E). As discussed above, the effective concentration range for this biphasic trend is significantly extended in the presence of cofilin. However, this property of CAP1-CT has another side to it – that is, a much larger amount of it greatly accelerates the disassembly of actin filament exclusively at the barbed end (K.M. and I.Y., unpublished). This effect argues for the presence of an interaction between CAP1-CT and the barbed end. Second, CAP1 increases the rate of subunit release at the pointed end of the actin filament and enhances the more potent, analogous effect of cofilin. The N-terminal domain primarily contributes to this activity of CAP1 (Fig. 5B), and the C-terminal domain augments it, probably by recycling cofilin and/or the N-terminal domain of CAP1. Third, CAP1 accelerates nucleotide exchange on G-actin to regenerate readily polymerizable ATP-actin (Fig. 5C,D). CAP1-CT is essentially responsible for this activity. CAP1-NT was found to play an interesting role in this activity. Although it had no apparent effect on nucleotide exchange in the absence of cofilin, it was found to relieve the inhibitory effect of cofilin on nucleotide exchange only when CAP1-CT was present together. This finding sheds light on the physiological significance of the reported interaction between the N- and C-terminal domains within CAP1 and CAP2 molecules (Hubberstey et al., 1996). The pronounced effect of CAP1 on cofilin-induced acceleration of actin turnover would be achieved through the integration of the four activities of CAP1, which involves a coordinated interplay between its N- and C-terminal domains.

Fragmentation of actin filaments affects actin dynamics. Our analysis refutes the possibility that CAP1 or its domains sever actin filaments in the absence of cofilin. At this time, however, we cannot completely exclude the possibility that CAP1 enhances severing by cofilin. If such enhancement does indeed occur, we think it is merely an indirect effect of CAP1. After released from the pointed end of the filament, the ADP-actin-cofilin complex should dissociate much faster in the presence of CAP1. As a result, released cofilin may make a second attack on F-actin to sever filaments again.

As mentioned above, cofilin strongly inhibits the nucleotide exchange of ADP-actin. This property of cofilin probably impedes the turnover of actin filaments. Any factor that stimulates dissociation of the ADP-actin monomer from cofilin should promote actin turnover. Higher eukaryotes possess cofilin kinases (including LIM kinases), which inhibit actin-binding by cofilin through phosphorylation (Arber et al., 1998; Yang et al., 1998; Smertenko et al., 1998; Lian et al., 2000; Aizawa et al., 2001). Phosphoinositides, which are present even in lower eukaryotes, also prevent cofilin from binding to actin (Yonezawa et al., 1990; Iida et al., 1993; Aizawa et al., 1995). It is thought that cofilin-kinases and/or phosphoinositides might promote the dissociation of the cofilin-actin complex. Our study identified CAP1 as a more promising factor to have such a property. CAP1 is perhaps better suited for this role because it relieves the inhibitory effect of cofilin on nucleotide exchange on G-actin via the interplay of its N- and C-terminal domains. In addition, its C-terminal domain is capable of stimulating nucleotide exchange on its own. Therefore, CAP1 effectively recycles both actin and cofilin for rapid actin turnover.

Freeman et al. (Freeman et al., 2000) recently reported that the microinjection of CAP1 into fibroblasts stimulated the formation of actin filaments in the cytoplasm and proposed a possible role of CAP1 in promoting actin assembly in vivo. Although they did not uncover how CAP1 could promote the actin assembly in cells, our present study will help interpret their observation.

Functional similarity and difference with profilin

In *S. cerevisiae*, loss of function of the C-terminal domain of CAP is compensated by profilin, a small actin-binding protein, and the cells lacking profilin are phenotypically similar to those lacking CAP-CT (Vojtek et al., 1991). In addition, expression of both mRNA and protein of CAP is remarkably upregulated in profilin-minus cells of *Dictyostelium* (Gottwald et al., 1996). The role of profilin in actin turnover has been studied in detail (Pring et al., 1992; Pantaloni and Carlier, 1993; Perelroizen et al., 1996; Selden et al., 1999; Wolven et al., 2000). Profilin accelerates nucleotide exchange on G-actin and facilitates subunit addition onto the barbed ends. Thus, profilin promotes the turnover of actin filaments in a similar manner to CAP1 and exhibits synergy with cofilin as well (Didry et al., 1998). In spite of these studies, CAP homologues have long been regarded as mere inhibitors of actin polymerization. CAP1-CT actually resembles profilin in its activity as revealed by our study. In reality, this similarity probably underlies the observed suppression of Δ*CAP-CT* by profilin in yeast. In clear contrast to CAP1, profilin does not associate with the cofilin-actin complex and thus cannot directly dissociate the complex. In other words, profilin does not possess CAP1-NT-like activity. Hence, CAP1 is unique in having a cofilin-dependent activity in its N-terminal domain.

Possible cellular regulators of CAP1 function

Internal proline-rich regions of CAP homologues have been reported to interact with several proteins implicated in cytoskeletal regulation (Freeman et al., 1996; Lila and Drubin, 1997). In addition, CAP homologues contain a sequence related to a verprolin homology domain, which is part of the WASP-family of actin-binding proteins (Baum et al., 2000).

These regions may also affect the modulation of actin dynamics by CAP homologues or cofilin in living cells.

Moreover, CAP is a component of the Ras signalling pathway in *S. cerevisiae*. In response to glucose, Ras activates Cyr1, an adenylyl cyclase, to stimulate cAMP synthesis and promote cell growth. CAP exists as a complex with Cyr1 and aids Ras signalling (Field et al., 1990; Fedor-Chaiken et al., 1990). The N-terminal region of CAP mediates its binding to Cyr1, and this association generates a second binding site for Ras (Shima et al., 2000). To date, relevant mammalian targets of CAP1-NT or CAP2-NT have not been identified, although human CAP1 may bind to yeast adenylyl cyclase in fission yeast (Yu et al., 1994). This is the first study to identify a physiologically relevant mammalian target: the cofilin-actin complex. However, it is likely that the interaction between CAP1 and actin-cofilin may be regulated by an unidentified partner of CAP1-NT, possibly a Ras/Cyr1-like signalling molecule.

We are grateful to H. Aizawa and K. Iida (our laboratory) for their valuable discussion and encouragement. We also thank A. Fujisawa-Sehara and H. Masai for their encouragement and support. This work was supported by a Grant-in-Aid for Specially Promoted Research (09101002) from the Ministry of Education, Science and Culture of Japan.

References

Aizawa, H., Sutoh, K., Tsubuki, S., Kawashima, S., Ishii, A. and Yahara, I. (1995). Identification, characterization, and intracellular distribution of cofilin in *Dictyostelium discoideum*. *J. Biol. Chem.* **270**, 10923-10932.

Aizawa, H., Katadae, M., Maruya, M., Sameshima, M., Murakami-Murofushi, K. and Yahara, I. (1999). Hyperosmotic stress-induced reorganization of actin bundles in *Dictyostelium* cells over-expressing cofilin. *Genes to Cells* **4**, 311-324.

Aizawa, H., Wakatsuki, S., Ishii, A., Moriyama, K., Sasaki, Y., Ohashi, K., Sekine-Aizawa, Y., Sehara-Fujisawa, A., Mizuno, K., Goshima, Y. and Yahara, I. (2001). Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. *Nat. Neurosci.* **4**, 367-373.

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* **393**, 805-809.

Baum, B., Li, W. and Perrimon, N. (2000). A cyclase-associated protein regulates actin and cell polarity during *Drosophila* oogenesis and in yeast. *Curr. Biol.* **10**, 964-973.

Benlali, A., Draskovic, I., Hazelett, D. J. and Treisman, J. E. (2000). *act up* controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the *Drosophila* eye disc. *Cell* **101**, 271-281.

Blanchoin, L. and Pollard, T. D. (1998). Interaction of actin monomers with *Acanthamoeba* actophorin (ADF/cofilin) and profilin. *J. Biol. Chem.* **273**, 25106-25111.

Brands, J. H., Maassen, J. A., van Hemert, F. J., Amons, R. and Moeller, W. (1986). The primary structure of the alpha subunit of human elongation factor 1: Structural aspects of guanine-nucleotide-binding sites. *Eur. J. Biochem.* **155**, 167-171.

Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H. and Pantaloni, D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.* **136**, 1307-1322.

Chen, H., Bernstein, B. W. and Bamburg, J. R. (2000). Regulating actin-filament dynamics in vivo. *Trends Biochem. Sci.* **25**, 19-23.

Didry, D., Carlier, M. F. and Pantaloni, D. (1998). Synergy between actin depolymerizing factor/cofilin and profilin in increasing actin filament turnover. *J. Biol. Chem.* **273**, 25602-25611.

Duh, F.-M., Latif, F., Weng, Y., Geil, L., Modi, W., Stackhouse, T., Matsumura, F., Duan, D. R., Linehan, W. M., Lerman, M. I. and Gnarra, J. R. (1994). cDNA cloning and expression of the human homolog of the sea urchin fascin and *Drosophila* singed genes which encodes an actin-bundling protein. *DNA Cell Biol.* **13**, 821-827.

Fedor-Chaiken, M., Deschenes, R. J. and Broach, J. R. (1990). *SRV2*, a gene required for RAS activation of adenylate cyclase in yeast. *Cell* **61**, 329-340.

Field, J., Vojtek, A., Ballester, R., Bolger, G., Colicelli, J., Ferguson, K., Gerst, J., Kataoka, T., Michaeli, T. and Powers, S. (1990). Cloning and characterization of CAP, the *S. cerevisiae* gene encoding the 70 kd adenylyl cyclase-associated protein. *Cell* **61**, 319-327.

Freeman, N. L., Chen, Z., Horenstein, J., Weber, A. and Field, J. (1995). An actin monomer binding activity localizes to the carboxyl-terminal half of the *Saccharomyces cerevisiae* cyclase-associated protein. *J. Biol. Chem.* **270**, 5680-5685.

Freeman, N. L., Lila, T., Mintzer, K. A., Chen, Z., Pahk, A. J., Ren, R., Drubin, D. G. and Field, J. (1996). A conserved proline-rich region of the *Saccharomyces cerevisiae* cyclase associated protein binds SH3 domains and modulates cytoskeletal localization. *Mol. Cell. Biol.* **16**, 548-556.

Freeman, N. L. and Field, J. (2000). Mammalian homolog of the yeast cyclase associated protein, CAP/Srv2p, regulates actin filament assembly. *Cell Motil. Cytoskeleton* **45**, 106-120.

Gerst, J. E., Ferguson, K., Vojtek, A., Wigler, M. and Field, J. (1991). CAP is a bifunctional component of the *Saccharomyces cerevisiae* adenylyl cyclase complex. *Mol. Cell. Biol.*, **11**, 1248-1257.

Gieselmann, R. and Mann, K. (1992). ASP-56, a new actin sequestering protein from pig platelets with homology to CAP, an adenylate cyclase-associated protein from yeast. *FEBS Lett.* **298**, 149-153.

Gill, S. C. and von Hippel, P. H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319-326.

Gottwald, U., Brokamp, R., Karakesisoglou, I., Schleicher, M. and Noegel, A. A. (1996). Identification of a cyclase-associated protein (CAP) homologue in *Dictyostelium discoideum* and characterization of its interaction with actin. *Mol. Biol. Cell* **7**, 261-272.

Hubberstey, A., Yu, G., Loewith, R., Lakusta, C. and Young, D. (1996). Mammalian CAP interacts with CAP, CAP2, and actin. *J. Cell Biochem.* **61**, 459-466.

Iida, K., Matsumoto, S. and Yahara, I. (1992). The KKRKK sequence is involved in heat shock-induced nuclear translocation of the 18-kDa actin-binding protein, cofilin. *Cell Struct. Funct.* **17**, 39-46.

Iida, K., Moriyama, K., Matsumoto, S., Kawasaki, H., Nishida, E. and Yahara, I. (1993). Isolation of a yeast essential gene, *COF1*, that encodes a homologue of mammalian cofilin, a low-M(r) actin-binding and depolymerizing protein. *Gene* **124**, 115-120.

Iida, K. and Yahara, I. (1999). Cooperation of two actin-binding proteins, cofilin and Aip1, in *Saccharomyces cerevisiae*. *Genes to Cells* **4**, 21-32.

Kouyama, T. and Mihashi, K. (1981). Fluorimetry study of *N*-(1-pyrenyl) iodoacetamide labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* **114**, 33-38.

Lappalainen, P. and Drubin, D. G. (1997). Cofilin promotes rapid actin filament turnover in vivo. *Nature* **388**, 78-82.

Lian, J. P., Marks, P. G., Wang, J. Y., Falls, D. L. and Badwey, J. A. (2000). A protein kinase from neutrophils that specifically recognizes Ser-3 in cofilin. *J. Biol. Chem.* **275**, 2869-2876.

Lila, T. and Drubin, D. G. (1997). Evidence for physical and functional interactions among two *Saccharomyces cerevisiae* SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol. Biol. Cell* **8**, 367-385.

McGough, A., Pope, B., Chiu, W. and Weeds, A. (1997). Cofilin changes the twist of F-actin: implications for actin filament dynamics and cellular function. *J. Cell Biol.* **138**, 771-781.

Mintzer, K. A. and Field, J. (1994). Interactions between adenylyl cyclase, CAP and RAS from *Saccharomyces cerevisiae*. *Cell Signal.* **6**, 681-694.

Mitchison, T. J. and Cramer, L. P. (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.

Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K. and Yahara, I. (1990). Destrin, a mammalian actin-depolymerizing protein, is closely related to cofilin. Cloning and expression of porcine brain destrin cDNA. *J. Biol. Chem.* **265**, 5768-5773.

Moriyama, K., Iida, K. and Yahara, I. (1996). Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. *Genes to Cells* **1**, 73-86.

Moriyama, K. and Yahara, I. (1999). Two activities of cofilin, severing and accelerating directional depolymerization of actin filaments, are affected differentially by mutations around the actin-binding helix. *EMBO J.* **18**, 6752-6761.

Nishida, E. (1985). Opposite effects of cofilin and profilin from porcine brain on rate of exchange of actin-bound adenosine 5′-triphosphate. *Biochemistry* **24**, 1160-1164.

Nishida, Y., Shima, F., Sen, H., Tanaka, Y., Yanagihara, C., Yamawaki-Kataoka, Y., Kariya, K. and Kataoka, T. (1998). Coiled-coil interaction of N-terminal 36 residues of cyclase-associated protein with adenylyl cyclase is sufficient for its function in *Saccharomyces cerevisiae* ras pathway. *J. Biol. Chem.* **273**, 28019-28024.

Noegel, A. A., Rivero, F., Albrecht, R., Janssen, K. P., Kohler, J., Parent, C. A. and Schleicher, M. (1999). Assessing the role of the ASP56/CAP homologue of *Dictyostelium discoideum* and the requirements for subcellular localization. *J. Cell Sci.* **112**, 3195-3203.

Okada, K., Obinata. T. and Abe, H. (1999). XAIP1: a *Xenopus* homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins. *J. Cell Sci.* **112**, 1553-1565.

Pantaloni, D. and Carlier, M. F. (1993). How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* **75**, 1007-1014.

Perelroizen, I., Didry, D., Christensen, H., Chua, N. H. and Carlier, M. F. (1996). Role of nucleotide exchange and hydrolysis in the function of profilin in actin assembly. *J. Biol. Chem.* **271**, 12302-12309.

Pring, M., Weber, A. and Bubb, M. R. (1992). Profilin-actin complexes directly elongate actin filaments at the barbed end. *Biochemistry* **31**, 1827-1836.

Rodal, A. A., Tetreault, J. W., Lappalainen, P., Drubin, D. G. and Amberg, D. C. (1999). Aip1p interacts with cofilin to disassemble actin filaments. *J. Cell Biol.* **145**, 1251-1264.

Rosenblatt, J., Agnew, B. J., Abe, H., Bamburg, J. R. and Mitchison, T. J. (1997). *Xenopus* actin depolymerizing factor/cofilin (XAC) is responsible for the turnover of actin filaments in *Listeria monocytogenes* tails. *J. Cell Biol.* **136**, 1323-1332.

Rosenblatt, J. and Mitchison, T. J. (1998). Actin, cofilin and cognition. *Nature* **393**, 739-740.

Selden, L. A., Kinosian, H. J., Estes, J. E. and Gershman, L. C. (1999). Impact of profilin on actin-bound nucleotide exchange and actin polymerization dynamics. *Biochemistry* **38**, 2769-2778.

Shima, F., Okada, T., Kido, M., Sen, H., Tanaka, Y., Tamada, M., Hu, C. D., Yamawaki Kataoka, Y., Kariya, K. and Kataoka, T. (2000). Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. *Mol. Cell. Biol.* **20**, 26-33.

Smertenko, A. P., Jiang, C. J., Simmons, N. J., Weeds, A. G., Davies, D. R. and Hussey, P. J. (1998). Ser6 in the maize actin-depolymerizing factor, ZmADF3, is phosphorylated by a calcium-stimulated protein kinase and is essential for the control of functional activity. *Plant J.* **14**, 187-193.

Suzuki, K., Nishihata, J., Arai, Y., Honma, N., Yamamoto, K., Irimura, T. and Toyoshima, S. (1995). Molecular cloning of a novel actin-binding protein, p57, with a WD repeat and a leucine zipper motif. *FEBS Lett.* **364**, 283-288.

Swiston, J., Hubberstey, A., Yu, G. and Young, D. (1995). Differential expression of CAP and CAP2 in adult rat tissues. *Gene* **165**, 273-277.

Vojtek, A., Haarer, B., Field, J., Gerst, J., Pollard, T. D., Brown, S. and Wigler, M. (1991). Evidence for a functional link between profilin and CAP in the yeast *S. cerevisiae*. *Cell* **66**, 497-505.

Wang, Y. L. and Taylor, D. L. (1981). Exchange of 1,N^6-etheno-ATP with actin-bound nucleotides as a tool for studying the steady-state exchange of subunits in F-actin solutions. *Proc. Natl. Acad. Sci. USA* **8**, 5503-5507.

Weber, A., Pennise, C. R. and Pring, M. (1994). DNase I increases the rate constant of depolymerization at the pointed (–) end of actin filaments. *Biochemistry* **33**, 4780-4786.

Wolven, A. K., Belmont, L. D., Mahoney, N. M., Almo, S. C. and Drubin, D. G. (2000). In vivo importance of actin nucleotide exchange catalyzed by profilin. *J. Cell Biol.* **150**, 895-904.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393**, 809-812.

Yonezawa, N., Nishida, E., Iida, K., Yahara, I. and Sakai, H. (1990). Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. *J. Biol. Chem.* **265**, 8382-8386.

Yu, G., Swiston, J. and Young, D. (1994). Comparison of human CAP and CAP2, homologs of the yeast adenylyl cyclase-associated proteins. *J. Cell Sci.* **107**, 1671-1678.

Zelicof, A., Protopopov, V., David, D., Lin, X. Y., Lustgarten, V. and Gerst, J. E. (1996). Two separate functions are encoded by the carboxyl-terminal domains of the yeast cyclase-associated protein and its mammalian homologs. Dimerization and actin binding. *J. Biol. Chem.* **271**, 18243-18252